Morphological determinants of carrier frequency signal in katydids (Orthoptera): a comparative analysis using biophysical evidence

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Abstract

Male katydids produce mating calls by stridulation using specialized structures on the forewings. The right wing (RW) bears a scraper connected to a drum-like cell known as the mirror and a left wing (LW) that overlaps the RW and bears a serrated vein on the ventral side, the stridulatory file. Sound is generated with the scraper sweeping across the file, producing vibrations that are amplified by the mirror. Using this sound generator, katydids exploit a range of song carrier frequencies (CF) unsurpassed by any other insect group, with species singing as low as 600 Hz and others as high as 150 kHz. Sound generator size has been shown to scale negatively with CF, but such observations derive from studies based on few species, without phylogenetic control, and/or using only the RW mirror length. We carried out a phylogenetic comparative analysis involving 94 species of katydids to study the relationship between LW and RW components of the sound generator and the CF of the male's mating call, while taking into account body size and phylogenetic relationships. The results showed that CF negatively scaled with all morphological measures, but was most strongly related to components of the sound generation system (file, LW and RW mirrors). Interestingly, the LW mirror (reduced and non-functional) predicted CF more accurately than the RW mirror, and body size is not a reliable CF predictor. Mathematical models were verified on known species for predicting CF in species for which sound is unknown (e.g. fossils or museum specimens).

Keywords: Stridulation, sound production, insect, bioacoustics, fossil, body size.

Introduction

Tettigoniidae, more commonly known as katydids or bush crickets, are insects known for their acoustic communication. Males produce calling songs to attract a mate using specialized structures on their forewings (Stumpner *et al.*, 2013). This sound production system comprises a serrated vein, also known as the stridulatory file, on the ventral part of the left wing (LW), which is passed across a sharp lobe or scraper on the anal margin of the right wing (RW) producing vibrations that are amplified by a membrane on the RW known as the mirror (Bailey, 1970; Morris, 1999; Heller & Hemp, 2014). The mirror is a delicate membrane stretched between a closed section of raised veins, which helps to radiate and amplify the sounds produced by the scraper and the file (Fig. 1) (Broughton, 1964; Bailey, 1967; Montealegre-Z & Postles, 2010). Katydid males possess mirror cells on both wings however, while the mirror on the RW is functional in most species, the LW mirror (the wing where the active file resides) is usually atrophied and damped to vibrations (Montealegre-Z & Postles, 2010; Montealegre-Z, 2012; Sarria-S *et al.*, 2016; Chivers *et al.*, 2017). Although, in some species, small wing cells associated with the mirror do exhibit vibrational properties during sound production (Sarria-S et al., 2016).

Using this sound generating mechanism, the range of song carrier frequencies produced by tettigonid species ranges from as low as 600 Hz (in *Tympanophyllum arcufolium*) to as high as 150 kHz (*Supersonus aequoreus*) (Heller, 1988; Morris *et al.*, 1994; Heller, 1995; Montealegre-Z *et al.*, 2006; Sarria-S *et al.*, 2014). Across insects, no other group produces such a wide range of acoustic communication signals using a single mechanism.

The structures specialized for sound production in animals usually scale with body size and with sound carrier frequency (CF) (Irschick *et al.*, 2014). CF is defined as the centre

61 frequency at which spectral energy reaches its maximum. This relationship can also be
62 observed in the sound generator apparatus of katydids where it was shown that any linear
63 dimension of the structures involved in sound production (e.g., the RW mirror, and the
64 stridulatory file) scales with the CF of the male's call (Morris & Pipher, 1967; Bailey, 1970;
65 Sales & Pye, 1974; Heller, 1995; Montealegre-Z, 2009). Although the LW mirror is usually
66 reduced and non-functional, in some species certain small wing cells associated to this mirror
67 vibrate in some species (Sarria-S et al., 2016).

68 Researchers have studied various forms of morphological scaling of the stridulatory
69 apparatus to identify the factors that affect the CF in singing katydids. Using two species of
70 katydid, Morris & Pipher (1967), based on Broughton's (1964) analysis suggested that the
71 RW mirror frame in the katydid tegmina could be modeled as vibrating cantilever to predict
72 CF. Bailey (1970) included the mirror measurements of two more species of Conocephalinae,
73 which he fitted in Morris & Pipher (1967) linear model, and concluded that the mirror frame
74 dimensions of the species he studied (*Ruspolia nitidula*) fitted this plot despite the fact that it
75 differed substantially from the *Conocephalus* species used by Morris & Pipher. Sales & Pye
76 (1974) in a larger review reinforced these two previous studies adding data for 15 more
77 species. Bailey's (1970) and Sales & Pye's (1974) analyses showed that most of the points
78 (originated from mirror frame length) fell close to Morris & Pipher's (1967) fitted line. Any
79 change in CF is explained by a significant amount of mirror dimension (R^2=0.99, P<0.01,
80 $\mathrm{Ln}f_o = 0.49\ \mathrm{Ln}l^{-2}$, regression obtained from extrapolated data points in Sales & Pye plotted
81 chart). Sales & Pye (1974) concluded that Morris & Pipher's assumption on similar physical
82 properties of the mirror, was likely to be true. In a different approach, Rust et al. (1999)
83 studied 11 species of katydid to investigate the relationship of the entire stridulatory field of
84 the LW, including mirror and surrounding areas, and the CF of the song to predict the
85 frequency that an extinct katydid would sing at. They found a negative correlation between

the stridulatory area and CF. None of the above analyses, however, took into account phylogenetic correction of the data, because such methods were not available at the time. Montealegre-Z (2009) produced a first comparative analysis across 58 species of katydids, reporting allometric correlations between several calling song acoustic parameters and morphological structures, including body size based on the method of Independent Contrast (Garland *et al.*, 1992). However, more rigorous phylogenetic comparative methods have not been applied, and a larger dataset is now available.

From the various morphological variables used by Montealegre-Z (2009), mirror scaling was based on the length of the frame, as in the cantilever model (Morris & Pipher, 1967; Bailey, 1970; Sales & Pye, 1974). However, it was recently demonstrated in some species that not only the RW mirror is involved in sound radiation, but two large cells adjacent to the mirror (called the harp and the neck, Fig. 1), play a role as well (Montealegre-Z & Postles, 2010; Sarria-S et al., 2016; Chivers et al., 2017). While the right mirror features prominently in sound radiation, the neck and harp show a considerable influence, especially when the handle (the vein connecting the mirror and harp) is thin, and must be taken into account as active wing areas devoted to sound production (see Fig. 1). In fact, early work by Bailey (1970) shows sound radiation maps of the wings of *Ruspolia nitidula*, which suggest that the wing cells surrounding the mirror are acoustically active.

For different reasons researchers have been interested in the relation of body size and CF. For example, in sexual selection studies of acoustic species, female can assess male size/quality based on their carrier frequencies (Wedell & Sandberg, 1995). Many researchers have reported that the frequency produced by katydids is inversely related to their body size (Wedell & Sandberg, 1995; Morris, 2008; Anichini *et al.*, 2017) meaning as a katydid's size increases the CF of its song will decrease. And recent work on katydids suggest that wing

area seems to be under positive sexual selection, as the relationship of wing size on male body size proves to be positive (Anichini et al., 2017).

The aim of this research is to produce a comparative analysis between the morphology of both the LW and RW mirrors (and, if applicable, the associated cells), the file, and the male CF using a large data set of 94 tettigonid species, while taking in to account body size and phylogeny. We predict that both the left and right mirror area are predictors of the CF and that a negative correlation exists between the area of the mirror and the frequency produced, meaning that smaller mirror areas will be associated with higher CF and larger mirrors to lower CF. Additionally, based on phylogenetically-corrected comparative methods, this research aims to identify morphological parameters that can be used in the construction of mathematical models to accurately predict CF. These models will enable researchers to infer the calling song frequencies from species where the actual recording of living animals is not possible, like in museum specimens or in extinct species only known from the fossil record. A multi-parameter model to predict CF will prove to be particularly relevant to palaeontology as fossil Orthoptera are rarely preserved in their complete form, often consisting of only one wing or isolated body parts.

Materials and Methods

Morphological data

We examined the sound generator structures in males of 94 different species of the family Tettigoniidae. Specimens belong to a research collection at the University of Lincoln, UK and samples were a mixture of alcohol-preserved, dry, and pinned specimens. Dry samples were re-hydrated in an insect-relaxing chamber (a hermetic plastic box filled with wet sand) for 24 hours. The purpose of this step was to soften the specimens, allowing for

easier manipulation of the sample without risk of shattering. This allowed for the extension of the wings (Gibb, 2014), making the forewing structures of both wings visible to be photographed. Wings of some animals were difficult to extend or to position and were removed using micro-scissors in order to get a clear picture of the mirror and therefore a more accurate area measurement. Using a VMS-004-LCD digital USB microscope camera (Veho Europe, Hampshire, UK), both the right and left forewings were photographed next to a scale (planar to the wing surface) with 0.5 mm increments using the image capture program Veho professional imaging (Veho Europe, Hampshire, UK). The surface area of the left and right mirrors was extracted using ImageJ v1.50g (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/). The wing vibrating areas measured were the cell contained in the mirror ring (for species with reduced neck and harps, e.g. most Pseudophyllinae, and Arachnoscelidina), and the area occupied by mirror-neck-harp (e.g., most conocephaloids), as we have evidence that these areas are also active in sound radiation in some species (see Fig. 1). The stridulatory file was measured as the length between the first tooth in the anal end to the last tooth of in the basal end from SEM pictures taken by the authors, except for those species indicated in Table S-1, which were obtained from published literature or from specimens borrowed from collections.

Measurements of pronotum and mid femora length were taken as indicator of body size and to control for body size effects (Gwynne & Bailey, 1988; Del Castillo & Gwynne, 2007; Montealegre-Z, 2009) using digital calipers (Poly-Cal Electronic Caliper W74-101-175, Fowler High Precision, Newton, MA, USA). The mid femur was used here as this appendage is not specialized for either sound production or sound reception, and will therefore not constitute a potentially biased structure that naturally scales with acoustic parameters. For *Chondroderella borneensis* and *Promeca* spp.), measurements were taken directly from photographs provided by Dr. Klaus-G. Heller.

159

160 **Acoustic data**

161 The CF data was obtained from a variety of sources, including published and
162 unpublished data (see Table S1). CF is defined as the peak of dominant energy observed in a
163 spectrum. Previously unpublished data was collected and provided by members of the
164 bioacoustics lab at the University of Lincoln. All recordings were attained in lab conditions
165 and involved ultrasound-sensitive equipment, and were digitized using sampling rates of
166 either 256, 512 or 1024·kilosamples·s^{-1}. Insects were placed in a mesh cage, hung from the
167 ceiling of a soundproof room, >1 m below the ceiling and >1 m above the floor, hence
168 mitigating against potential reflections. Sound recordings were obtained using a 1/8" Brüel &
169 Kjær Type 4138 condenser microphone, connected to a Brüel & Kjær 2633 preamplifier
170 (Brüel & Kjær, Nærum, Denmark). Data were stored on a notebook computer using an NI
171 USB-6259 board (National Instruments, Austin, TX, USA) and LabVIEW version 9 (32 bit)
172 2009 software interface (National Instruments, Austin, TX, USA).

173 **Statistical analysis**

174 To account for non-independence of residuals in models that include evolutionarily
175 related species (Felsenstein, 1985), we carried out a phylogenetic generalized least squares
176 approach (PGLS) between the log frequency and log morphology measurements (Pagel,
177 1999; Freckleton *et al.*, 2002). The pglmEstLambda function was used to identify the
178 maximum likelihood value of lambda, λ (Pagel, 1999; Orme *et al.*, 2009; Revell, 2010),
179 which measures the degree to which the covariance matrix (built as part of CAIC) follows a
180 Brownian model. λ can vary between 0 (no phylogenetic autocorrelation) and 1 (complete
181 phylogenetic autocorrelation). The phylogeny of the 94 species used was adapted from the
182 most recent katydid phylogeny (Mugleston *et al.*, 2013), which was built using molecular

183 sequence data for 6 genes from 135 taxa representing 16 of the 19 Tettigoniidae subfamilies
184 (see appendices 1 and 2).

185 All variables were log-transformed (using natural logarithm). We tested how CF
186 scaled with single morphological components (pronotum length, file length, right mirror area,
187 left mirror area and femur length). We present results from the PGLS along with results from
188 OLS (ordinary least squares) for comparison (Freckleton, 2009). Following this, we tested all
189 pair combinations of morphological variables. We assessed the best model based on AICc
190 values. Models with $\Delta AICc<5$ were considered broadly equivalent. For all models, residuals
191 were examined for heteroscedasticity and outliers. Analysis was carried out using R code
192 kindly provided by R.P. Freckleton (University of Sheffield, UK). All models were carried
193 out in R version 3.2.1 (R Core Team, see the R homepage), see references.

194 **Testing the models on control Species**

195 The accuracy of the predictive models was tested using morphological measurements
196 of species not used to construct the phylogenetic regression, but for which reliable call
197 recordings exist in our collection or in the literature. In this case, the morphological structure
198 was entered into the PGLS regression equations and the difference between actual and
199 expected CF was calculated. All values were transformed to be positive, irrespective of
200 whether higher or lower values were calculated. Recent evidence (Chivers et al., 2017),
201 suggests a functional role for the harp area alongside the mirror region of the wing. Therefore,
202 we additionally calculated the difference between actual and predicted CF of the right and
203 left mirror, with and without the harp region included. Lastly, we tested whether difference
204 (irrespective of direction) was correlated with actual CF using Pearson's correlations.
205 Significant values would suggest systematic biases in CF prediction. The species used to test
206 the models are listed in Table S-2.

Results

We measured morphological structures (mirror area, file length) of the sound generator in the left and right forewings coupled with pronotum and mid-femur lengths in 94 species of katydids to investigate allometric relations with calling song frequency. Log CF scaled allometrically with all morphological measures (Table 1), but was most strongly related to components of the sound generation system (file length, LW and RW mirrors; Fig. 2). Of these, LW and RW mirrors explained the greatest single amount of variation in the data (Table 1; Figure 2CD).

Diagnostic plots show that one species (*Tympanophyllum arcufolium*) was a significant outlier in the analysis of log frequency and pronotum length. Exclusion of this point changed the regression equations R^2 (Table 2), suggesting that pronotum length may be the least reliable of parameters (Fig. 2A). Based on AIC values, three models had reasonable support (Δ AICc<5; Table 2). Of these, the best model was the combination of file length and left mirror (Table 2). Interestingly, this model was not phylogenetically dependent (λ=0: Table 2), whereas there was stronger phylogenetic dependence on the model containing the right mirror.

Testing the models on control species

In order to establish which structure of the sound generator in the wings of katydids predicts the CF with lower error, we calculated CF in species that were not used in the PGLS analysis, for which CF values and wing anatomy were available (see Table 3). This analysis suggests that the best predictors of CF were the file length (mean difference between actual and predicted CF=1.83 kHz) and the left mirror area (mean difference between actual and predicted CF=1.25 kHz; Figure 4). Interesting, the RW mirror area performed poorly as a

predictor of CF, though this improved when harp and neck cells areas were included (Fig 3C). More specifically, it halved the mean estimated difference in real and predicted CF. Inclusion of the LW harp area did not appreciably improve the prediction of CF (Figure 4).

The difference between actual and predicted CF was positively correlated for the RW mirror (r_p=0.52, p=0.041) and the LW mirror + harp area (r_p=0.63, p<0.001), suggesting that they become less reliable at high frequencies. In contrast, no other feature showed any relationship (right mirror +harp: r_p=0.23, p=0.384; file length: r_p=0.16, p=0.549; left mirror: r_p=-0.34, p=0.197)

Discussion

This research shows a high level of predictability of CF from the structure of the stridulatory apparatus, even after correction for phylogenetic-dependence. Although all the studied morphological structures of the stridulatory apparatus in both LW and RW could be used to predict CF, those of the LW (mirror and stridulatory file) predict CF with a higher level of accuracy than vibratory areas of the RW (Fig. 4, Table 3). This finding was unanticipated because the right mirror has been traditionally known to be the main source of sound radiation across species and the focus of attention of researchers working in katydid stridulation (Broughton, 1964; Bailey, 1967; Morris & Pipher, 1967; Sales & Pye, 1974; Morris *et al.*, 1975), whilst the left mirror is usually damped or atrophied (Montealegre-Z, 2005; Montealegre-Z & Mason, 2005; Montealegre-Z & Postles, 2010; Montealegre-Z, 2012; Sarria-S et al., 2014; Sarria-S et al., 2016). This morphological asymmetry in the stridulatory fields of the LW and RW is one of the synapomorphic features of the family Tettigoniidae (Rust et al, 1999; Montealegre-Z, 2005). Hemp et al. (2015) studied the scaling of length and width of left (N=7 species) and right (N=4 species) mirrors as predictors of CF in *Afroanthracites* and *Afroagraecia* species. They found a high correlation for the length of left

and right mirror but lower for the width. In particular, the left mirror width had a lower correlation due to only one species with real asymmetrical tegmina *Afroagraecia brachyptera*. In our study, we use mirror area to predict CF. In the case of species with reduced or atrophied left mirrors, simple linear dimensions may not therefore capture accurately the mirror shape or size. For example, in *Afroagraecia brachyptera* the left mirror is more triangular in shape making width dimensions harder to measure compared to other species (see Fig. 11: Hemp et al. 2015). Hence, we believe mirror area is a better measure to account for shape variance in mirrors.

The fact that the left mirror predicts CF more accurately than the right mirror could be explained by the fact that although the right stridulatory field is heavily involved in sound production, it exhibits more variation across species than the left stridulatory field. For example, in several species not only does the right mirror radiate sound but also the adjacent cells (the neck and the harp, Fig. 1AB) exhibit high levels of deformation during resonant vibration (Montealegre-Z & Postles, 2010; Chivers et al., 2017; Jonsson *et al.*, 2017). In other species (e.g., many Pseudophyllinae, Fig. 1C) the right stridulatory area is limited to the mirror itself, while the neck and harps are atrophied or reduced to be acoustically inactive (Montealegre-Z, 2005; Montealegre-Z, 2012) (see Fig 1C). Such reduction of the sound radiating area to a single mirror ring seems to be associated with levels of sound purity or tonality (narrow-band spectra). Species with pure tone calls usually have a single isolated right mirror (Morris & Beier, 1982; Morris *et al.*, 1989; Montealegre-Z & Morris, 1999), while those with more active sound radiating cells tend to emit more broadband frequency calls (Montealegre-Z, 2005; Montealegre-Z & Mason, 2005; Gu *et al.*, 2012; Chivers et al., 2017). The large amount of variation in spectral quality observed in Tettigoniidae, a family in which many species are usually regarded as broadband sound emitters, may partly be explained by multiple active sound radiating cells. Inclusion of the neck and harp areas plus

right mirror was found to provide a better prediction of CF than right mirror on its own, perhaps emphasizing the importance of these relatively unexplored areas on CF production.

The left mirror on the other hand has maintained a more conservative structure than the right mirror. While the left mirror is atrophied in most species studied so far (Montealegre-Z & Postles, 2010; Montealegre-Z, 2012; Sarria-S et al., 2014; Sarria-S et al., 2016; Chivers et al., 2017; unpublished data), the right mirror underwent selection pressures for frequency diversity based on diversified resonances. Although some left mirrors do contain extra vibrating areas, these remain small and inclusion of these has a minor impact on CF. In the absence of a laser Doppler vibrometer, acoustically functional cells in the left mirror could be identified usually by their level of transparency and by a conspicuous reduction in cross veins. In general, it is relatively uncommon to observe functional left mirrors, and even rarer to find an active left mirror, harp and neck across living katydid species. Nevertheless, in most *Conocephalus* spp. *Orchellimum* spp. *Afroanthracites* spp, *Phlugis* spp., *Neduba* spp. *Acanthacara* spp., *Decticus* spp., *Platycleis* spp. or *Tettigonia* spp., and in many species of *Saginae*, for example, the left mirror exhibits some level of specialization for sound radiation. Not surprisingly, males of these species tend to produce broadband calling songs (Morris & Pipher, 1967; Morris & Pipher, 1972; Pipher & Morris, 1974; Morris et al., 1975; Heller, 1988; Jatho *et al.*, 1992; Jatho *et al.*, 1994; Morris & Mason, 1995; Hemp *et al.*, 2015; Lemonnier-Darcemont et al., 2016). Even when the left stridulatory cell shows some levels of activity, high levels of mechanical asymmetry between both left and right wings are observed, with maximum amplitude exhibited by the right stridulatory area (Montealegre-Z & Mason, 2005; Montealegre-Z & Postles, 2010; Sarria-S et al., 2016; Chivers et al., 2017; Jonsson et al., 2017). This wing asymmetry seems to have been selected as a way to reduce acoustic interference between two wings and favoured the use of ultrasonic frequencies (Montealegre-Z, 2005; Gu et al., 2012). We believe that the LW mirror

has been under relatively lower selection pressures than the RW mirror, which means that although atrophied in many species, the left mirror has maintained a stronger allometric relationship to the sound production unit as a whole and as a consequence is a better predictor than the more variable right mirror.

The specific radiation resistance, and therefore its efficiency as a sound source, depends on the ratio between its diameter and the sound wavelength. The minimum source size for good source to-medium matching has a radius of about 1/4 of the fundamental wavelength if it is a dipole source, below this size the specific radiation resistance decreases more or less rapidly (Bennet-Clark, 1998). In almost all species of Tettigoniidae studied here, the dimensions of the sound radiator are smaller than the optimal size for sound radiation related to wavelength (see also Montealegre-Z, 2005); only in the species with the most ultrasonic calls is the area of radiating surface optimal. Bennet-Clark (1998) discussed these aspects and pointed out that it is difficult to study the problem of specific acoustic resistance in tettigoniids because these animals could have evolved secondary resonators in their forewings, pronotum and subalar spaces.

Applications of the mathematical models

The rationalization for this study was to produce predictive models that could eventually help to calculate the CF in extinct katydids or museum specimens that we can no longer determine the CF from (by either analysis of song recordings or from wing vibration measurements). Fossils may only be partial (rely on single body part, usually the wings) and the multiple models presented in this research allow a range of frequencies to be calculated, with varying levels of accuracy.

Our results suggest that the CF from fossilized males could be recovered with high degree of reliability using left mirror area and file length. However, our model of CF on file

length is reliable only in those species using CFs below 40 kHz (frequencies >40 kHz were not included in the test, see Table S2), which is ideal for fossils because it has been shown that Jurassic and Cretaceous species exploited low-frequency signals (Rust et al., 1999; Gu et al., 2012). The length of the stridulatory file and the number of teeth a file holds scale allometrically with body size, with larger files tending to possess more teeth (Montealegre-Z, 2005; Montealegre-Z, 2009; Anichini et al., 2017). Therefore it is not surprising that file length and other file attributes correlate with CF. For example, pure-tone species use a 1:1 relation between tooth strikes and the number of oscillations produced, i.e., more densely packed teeth in theory will produce more oscillations per unit time, thus higher frequencies. However above 40 kHz this 1:1 relationship between oscillations and teeth is not valid any more. At such elevated CFs species use discrete tooth-strikes delivered at elevated rates and speeds by scraper elasticity. The stridulatory file should have a minimum size to host the necessary number of teeth for paused discrete tooth strikes and produce a pulse train of decent duration (extreme high frequencies). Therefore, for extreme ultrasonic species it is more important to evolve files of effective lengths to host teeth with particular distribution to facilitate scraper deformation and elevated rates of tooth strikes. For instance, if the file of the highest frequency species (*Supersonus aequoreous*, singing at 150 kHz) is used to predict CF, the value obtained will be ca 60 kHz. For the reason, the mirrors are better predictors of CF in this case.

Recent robust molecular phylogeny suggests the Tettigoniidae could have emerged in late Jurassic (Song et al., 2015). However, modern forms of Tettigoniidae with differentiated mirrors and asymmetric stridulatory fields as one of the major features distinguishing them from other Ensifera, appear in the fossil record some 60 mya (Heads & Leuzinger, 2011; Rust et al., 1999; Greenwalt & Rust, 2014). This wing anatomy facilitates inferring CF from equations presented in Table 1. In fact, using the morphological features of the extinct

354 *Pseudotettigonia amoena* described in Rust et al. (1999), (only LW anatomy are reported),
355 we predict CFs of a) 10.5kHz (using file length), b) 12.6kHz (using LW mirror area only),
356 and c) 9.12 kHz (using LW mirror plus harp area) (Table 3). In this situation the CF values
357 obtained in a and c are very close; these results suggest that this species used a CF of about
358 10 kHz. But why did we use the mirror/harp and not the sole mirror as in the calculation?
359 Rust et al. (1999) reported that the wings of *P. amoena* seemed to have been more
360 symmetrical than those of modern forms, it is thus likely that the stridulatory field (mirror
361 and harp) of the LW was very active in sound production. This is supported by the wing
362 anatomy, which shows large mirror and harps, both connected by a thin handle vein,
363 characteristic of large functional stridulatory areas (as shown in Fig. 1B). Consequently,
364 using the entire area of mirror and harp is recommended here; in addition, the predicted CF
365 is in agreement with that obtain using the file. The envisaged CF (ca 10 kHz) is indeed about
366 3 kHz higher than the 7 kHz originally anticipated by Rust et al. (1999). Similar results were
367 obtained for *P. leona*, 11.0 kHz (using file length), 16.2 kHz (using LW mirror area only) and
368 11.1 kHz (using LW mirror plus harp area) (Table 3). CF inferred from file, and that inferred
369 from and mirror plus harp match at ~11 kHz. The predicted CF of 11 kHz is about 3.5 kHz
370 higher than originally calculated (6.5 kHz) by Greenwalt & Rust (2014). We also recalculated
371 CF in *Archaboilus musicus*, another extinct species from the middle Jurassic for which the
372 calling song was reconstructed a few years ago based on comparative methods and
373 phylogenetic control of 59 species, and mechanical principles of stridulation (Gu et al., 2012).
374 The original reconstruction by Gu et al. (2012) anticipated a CF of 6.4 kHz (Table 3), with
375 our new mathematical models, based on file dimension the CF dropped by 1.4 kHz to ca. 5.0
376 kHz, a CF used by most field crickets today (Michelsen, 1998; Bennet-Clark, 2003).

377 In extinct hagloids (including Haglidae and Prophalangopsidae) the mirrors on left
378 and right tegmina are not as well defined as in modern tettigonids and vibratory patterns of

379 the wings are variable (Chivers et al., 2017). Hence, using mirror dimension to estimate CF
380 proves challenging. In circumstances like this, a combination of inferred CFs based on file
381 length and vibrating areas (inferred from LDV recordings or by identifying cells without
382 cross veins as potential vibrating areas) could be useful. An example is shown using the relic
383 species *Cyphoderris monstrosa* (a Prophalangopsidae). Using the entire vibrating areas
384 (mirror plus harp and neck), and the stridulatory file, the anticipated CF closely matches the
385 call CF. The left mirror predicts a relatively accurate CF value of 13.6 kHz against 13.1 kHz,
386 Table 3). The wings of *Cyphoderris* are highly symmetrical, as in their extinct ancestors, and
387 in theory left and right wing anatomy should predict CF similarly. However, we noted that
388 using only the equation of CF on left mirror to extrapolate values of either left of right
389 mirrors, will produce better results. For more recent forms of katydids (e.g., Cenozoic) we
390 recommend using the mathematical models for left and right mirrors and file, when
391 applicable.

392 In summary, here we present different solutions to infer the carrier frequencies used
393 by extinct fossilized ensiferan specimens. Orthopteran fossils are not always available with
394 the entire body preserved, but certainly the wings are one of the body structures that
395 preserves with good detail; and fossilized wings are not always found in pairs or complete.
396 We provide mathematical models (Table 1) that could be used for specific situations to
397 satisfy the needs imposed by available incomplete specimens.

398 **Acknowledgments**

399 This project was fully funded by the Leverhulme Trust (grant RPG-2014-284). We thank
400 Klaus-G. Heller for providing photographs and morphological data for specimens not
401 available in our collections, and valuable comments to improve the manuscript. We are also
402 grateful of two anonymous reviewers.

403 **Conflict of interest**

404 The authors declare no conflict of interest.

405 **Supporting information**

406 Additional Supporting Information may be found online in the supporting information tab for
407 this article:

408 **Table S1** Morphological and acoustic data of 94 species of Tettigoniidae used in the analysis.

409 **Table S2** Testing the mathematical models proposed in this research.

410 **Appendix S1**: Text form of the phylogenetic tree 94 species of katydid used in this research.

411 **Appendix S2**: Phylogenetic tree 94 species of katydid used in this research.

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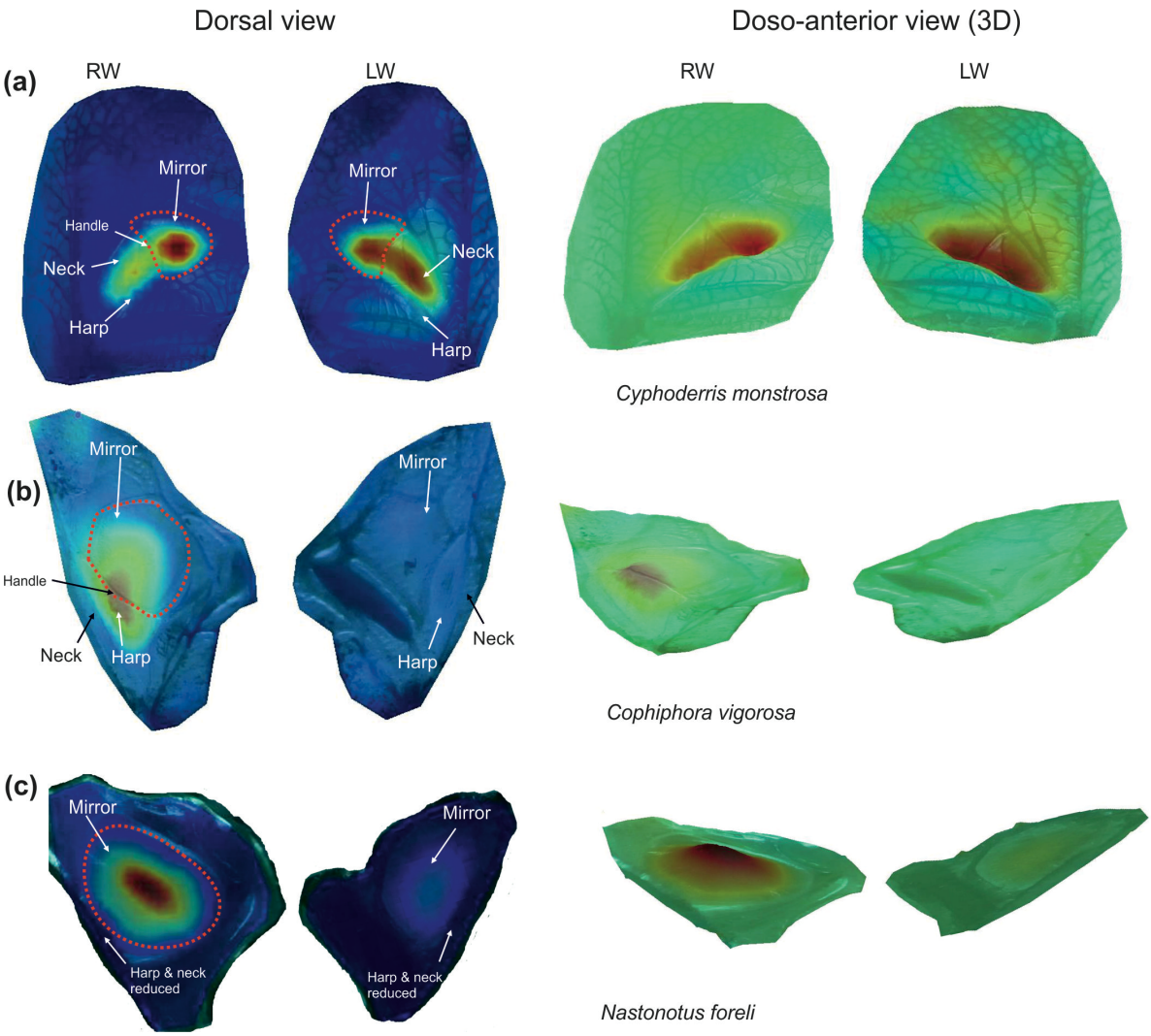
Figure captions

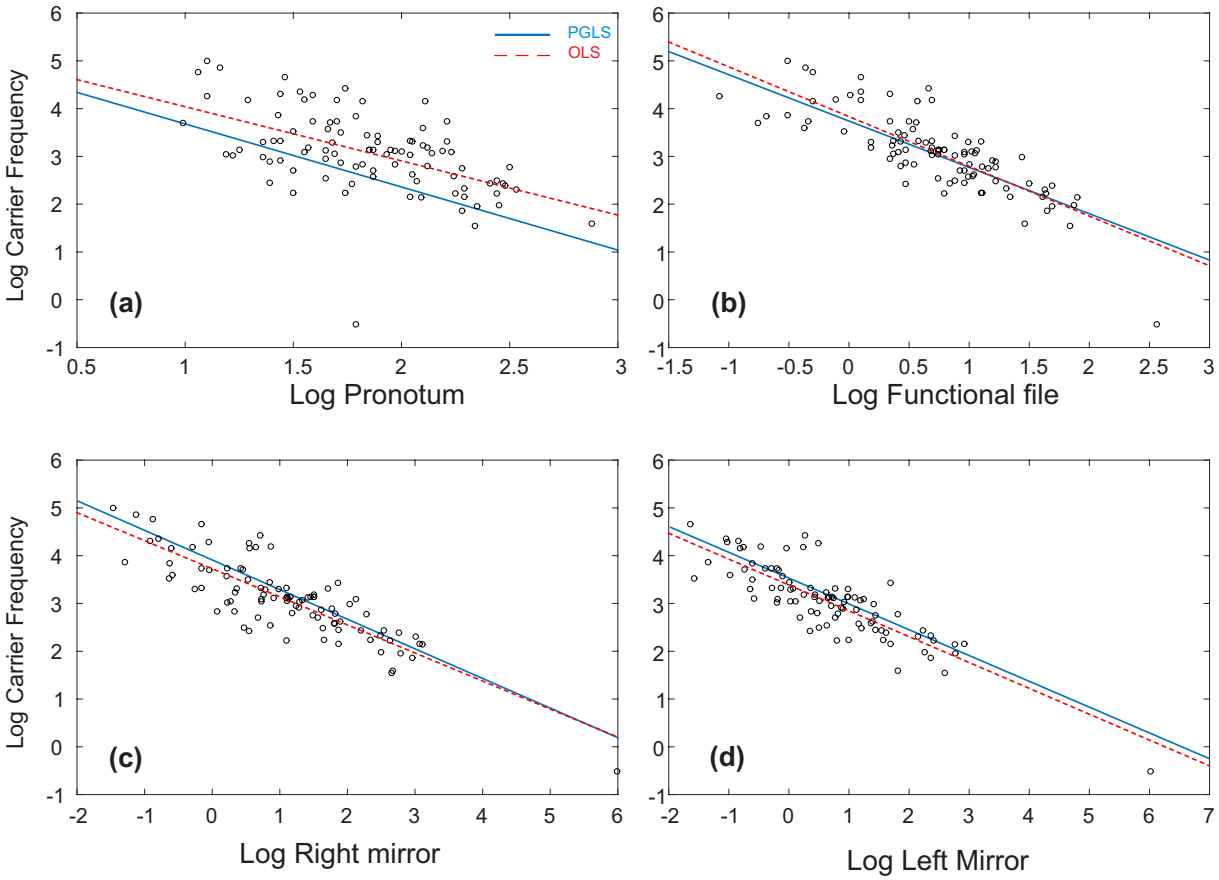
Fig. 1. Wing vibration maps of different species showing the major deflection areas of both wings (RW=right wing, LW= left wing) at the carrier frequency of the calling song. (a) *Cyphoderris monstrosa* (Prophalangopsidae). (b) *Copiphora vigorosa* (Conocephalinae). (c) *Nastonotus foreli* (Pseudophyllinae). Left column (dorsal view) shows the deflection maps of the wings highlighting the vibrational contribution of the wing cells named as 'neck' and 'harp' (a and b). Note that in c the mirror frame completely isolates the vibrations and deflections of the mirror membrane from the rest of the wing. The right column (dorso-anterior view) shows the 3D vibration of the wings to illustrate the connection between mirror and harp). In some species the elasticity of a thin vein known as the handle (a and b) extend vibration beyond the mirror frame. Such elasticity and thin connection between mirror and harp does not occur in c. The mirror frame is shown with a red dashed outline. a modified from Chivers et al. (2017); b modified from Sarria et al. (2016), c unpublished data).

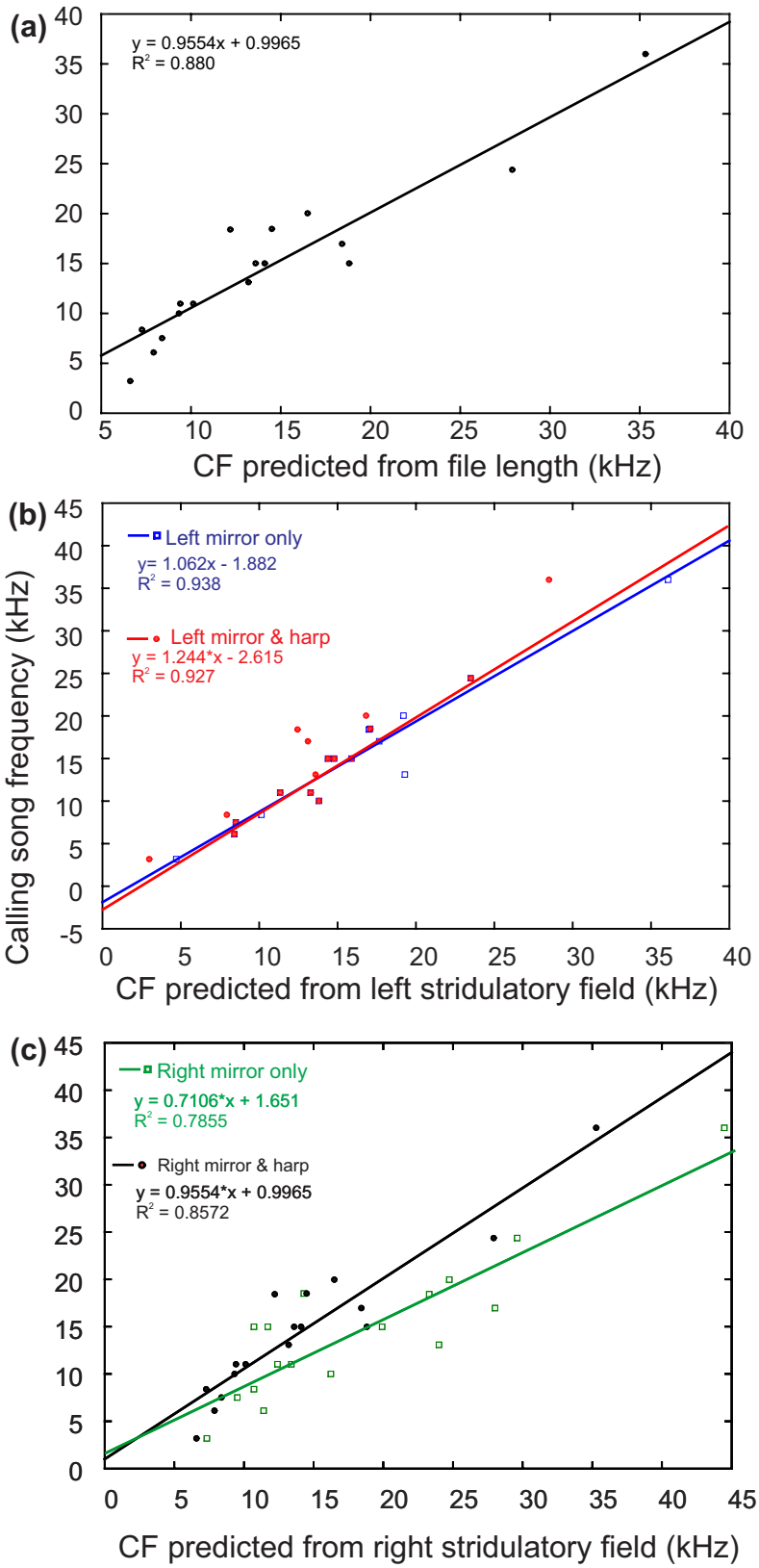
Fig. 2. Morphological predictors of carrier frequency. (a) Pronotum length as indicative of body size. (b) Stridulatory file length as predictor of CF. The functional file was used in katydids. However, for species with symmetric wings and two active stridulatory files (e.g. *Cyphoderris monstrosa*) any of the two files could be used. (c-d) Right mirror and left mirror areas as predictor of CF, respectively. All plots show both the regression lines of the Ordinary Least Squares (OLS) and Phylogenetic Generalized Least Squares (PGLS).

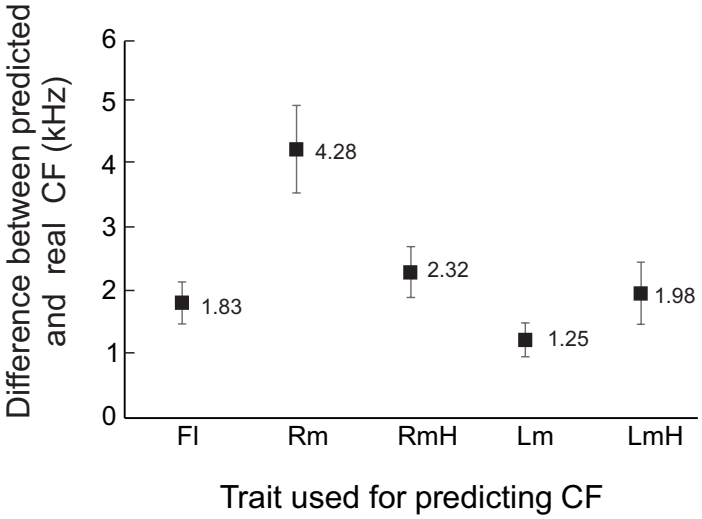
Fig. 3. Validation of the PGLS models using extant species not included in the analysis for which calling song CF and wing anatomy were available. (a) Song CF on CF predicted from file dimension. (b) Song CF on left stridulatory field area, using mirror area only (red trace) and the areas occupied by the mirror and adjacent harp (black outline, as shown in figure 1AB. (c) Song CF on right stridulatory field only (blue outline) and the area occupied by mirror and harp.

Fig. 4. Corroboration of the models. The plot shows the difference between predicted frequency and real CF using species not included in the comparative analysis. No difference (zero) indicated the predicted and real values are the same and the model is highly accurate. Fl= File length, Rm= Right mirror, RmH= Right mirror plus harp, Lm= Left mirror, LmH= Left mirror plus harp. The plot shows that the best predictors are Fl and Lm.


Dorsal view
Doso-anterior view (3D)
(a)
RW
LW
RW
LW
Mirror
Handle
Neck
Harp
Mirror
Neck
Harp
Cyphoderris monstrosa
(b)
Mirror
Handle
Neck
Harp
Mirror
Harp
Neck
Cophiphora vigorosa
(c)
Mirror
Harp & neck reduced
Mirror
Harp & neck reduced
Nastonotus foreli


PGLS
OLS
Log Carrier Frequency
(a)
Log Pronotum
(b)
Log Functional file
(c)
Log Right mirror
(d)
Log Left Mirror


(a)
y = 0.9554x + 0.9965
R² = 0.880
CF predicted from file length (kHz)
(b)
Left mirror only
y= 1.062x - 1.882
R² = 0.938
Left mirror & harp
y = 1.244*x - 2.615
R² = 0.927
Calling song frequency (kHz)
CF predicted from left stridulatory field (kHz)
(c)
Right mirror only
y = 0.7106*x + 1.651
R² = 0.7855
Right mirror & harp
y = 0.9554*x + 0.9965
R² = 0.8572
CF predicted from right stridulatory field (kHz)


Difference between predicted and real CF (kHz)
6
5
4
3
2
1
0
4.28
2.32
1.83
1.25
1.98
Fl
Rm
RmH
Lm
LmH
Trait used for predicting CF

582

414 **Table 1:** PGLS model outputs comparing log frequency in relation to different morphological features. Models show phylogenetically-correct
415 estimates and OLS estimates, Pagel's λ and R^2 for the models. * includes removal of single outlier

416

				Phylogenetically-controlled			**Non-phylogenetically controlled**		
Model	**Parameter**	**λ**	**R^2**	**B ± SE**	**t**	**p**	**B ± SE**	**t**	**p**
Midfemur length	Intercept	1.00	0.18	4.74±0.50	9.49	<0.001	4.33±0.46	9.49	<0.001
	Log midfemur length			-0.95±0.20	-4.68	<0.001	-0.58±0.21	-2.76	<0.001
Pronotum length	Intercept	1.00	0.31	5.00±0.44	11.38	<0.001	5.17±0.34	15.08	<0.001
	Log pronotum			1.32±0.20	-6.55	<0.001	-1.13±0.18	-6.21	<0.001
Left mirror area	Intercept	0.70	0.61	3.53±0.18	20.04	<0.001	3.86±0.05	69.74	<0.001
	Log left mirror			-0.54±0.06	-12.03	<0.001	-0.54±0.03	-15.67	<0.001
Right mirror area	Intercept	0.92	0.62	3.91±0.22	17.41	<0.001	3.72±0.06	60.36	<0.001
	Log right mirror			-0.62±0.05	-12.38	<0.001	-0.59±0.04	-15.27	<0.001
File length	Intercept	0.86	0.52	3.74±0.23	16.05	<0.001	3.83±0.07	51.75	<0.001
	Log file length			-0.97±0.10	-10.05	<0.001	-1.04±0.08	-13.49	<0.001
Pronotum length*	Intercept	0.98	0.42	5.00±0.33	15.10	<0.001	5.23±0.29	18.14	<0.001

417

418 **Table 2:** PGLS model outputs comparing log frequency in relation to pairs of morphological characters, of the three best models. Models show
419 phylogenetically-correct estimates and OLS estimates, Pagel's λ and R^2 for the models.

420

			Phylogenetically-controlled				Non-phylogenetically controlled		
Parameter	**λ**	**R^2**	**AICc**	**B ± SE**	**t**	**p**	**B ± SE**	**t**	**p**
Intercept	0	0.76	107.76	3.61±0.06	59.38	<0.001	3.60±-0.07	49.98	<0.001
Left mirror				-0.36±0.05	-7.18	<0.001	-0.36±0.06	-6.29	<0.001
File length				-0.46±0.09	-5.14	0.002	0.44±0.11	-3.87	0.002
Intercept	0.91	0.66	109.15	4.54±0.29	15.52	<0.001	4.25±0.22	18.92	<0.001
Right mirror				-0.53±0.05	-3.19	<0.001	0.53±0.04	-12.26	<0.001
Pronotum length				0.53±0.16	-9.77	0.017	-0.32±0.13	-2.44	0.017
Intercept	0.77	0.66	112.26	3.92±0.18	21.78	<0.001	3.81±0.07	58.52	<0.001
Right mirror				-0.46±0.08	-6.08	<0.001	-0.39±0.07	-5.33	<0.001
File length				-0.36±0.13	-2.75	0.002	-0.42±0.13	-3.12	0.002

Table 3. Testing the mathematical models proposed in this research on fossils. We venture CF inference from published fossil material, where authors tried to infer CF from using various allometry methods. †= extinct species. ‡= two values from LW and RW files, respectively. (*) CF inferred without phylogenetic control. (**) CF calculated controlling for phylogeny. (?) Unknown data. Tett.=Tettigoniidae. Proph. =Prophalangopsidae. RM= Right mirror, LM= Left mirror

Species	Family	CF of male's call (kHz)	File length (mm)	RM area (mm^2)	RM & harp area (mm^2)	LM area (mm^2)	LM & harp area (mm^2)	CF (kHz) from file	CF (kHz) from RM	CF (kHz) from RM & harp	CF (kHz) from LM	CF (kHz) from LM & harp	Source
Cyphoderris monstrosa	Proph.	13.1	3.14/3.3 ‡	9.76	9.76	9.77	9.77	13.9/13.2 ‡	24	16	19.3	13.6	(Chivers et al., 2017)
Pseudotettigonia amoena †	Tett.	7.0*	4.2	?	?	6.35	11.5	**10.5**	N/A	N/A	12.6	**9.12**	(Rust et al., 1999)
Pseudotettigonia leona †	Tett.	6.5*	4.2	?	?	3.96	8.13	**11**	N/A	N/A	16.22	**11.1**	(Greenwalt & Rust, 2014)
Archaboilus musicus †	Proph.	6.4**	9.34	?	?	?	?	4.99	N/A	N/A	N/A	N/A	(Gu et al., 2012)